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# Cholinergic Fiber Aberrations in Nucleus Basalis Lesioned Rat and Alzheimer's Disease

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GAYKEMA, R. P. A., C. NYAKAS, E. HORVATH, L. B. HERSH, C. MAJTENYI, AND P. G. M. LUITEN. *Cholinergic fiber aberrations in nucleus basalis lesioned rat and Alzheimer's disease*. NEUROBIOL AGING 13(3)441–448, 1992.—Innervation density and morphological aberrations of cholinergic fibers were studied with choline acetyltransferase (ChAT) immunocytochemistry and acetylcholinesterase (AChE) histochemistry in 30–35 month-old aged rats and rats with long-term bilateral lesions of the magnocellular basal nucleus (MBN). In addition, AChE histochemistry was performed on human cortical sections derived from autopsy brains of normal aged and Alzheimer's disease (AD) patients. A limited but variable number of morphological alterations were observed in ChAT-immunoreactive fibers in the cortex and the hippocampus of the aged control rats. The aged MBN-lesioned rats displayed a severely reduced number of cholinergic fibers in the denervated areas of the neocortex, whereas the surviving fibers showed a strongly increased number of aberrations. Fiber anomalies were also observed in the cortex of the aged human subjects and Alzheimer patients, the latter showing a higher incidence of such aberrations. Only a part of these distended profiles were seen in close association with senile plaques as detected in the AChE-stained material. These findings suggest that experimental MBN lesions combined with aging share with AD the induction of large quantities of fiber malformations. Implications of possible mechanisms in both conditions are discussed.

Rat	Cortex	Ibotenic acid lesions	Choline acetyltransferase	Acetylcholinesterase	Age-related deficit
Alzheimer's disease					

DEGENERATION of cholinergic neurons in the basal forebrain and of their ascending projections to the cerebral cortex, amygdala, and hippocampus is a major pathological event in Alzheimer's disease (AD) (10,35). Extensive animal model research on behavioral consequences of pharmacological blockade of cholinergic transmission and of lesioning of the basal forebrain cholinergic nuclei has provided evidence for a causal relationship between damage to the forebrain cholinergic systems and impairment in behavioral performance in memory tasks (see reviews in 25,29). These studies have supported the hypothesis that memory impairments in AD may in part be attributed to the decline in cortical cholinergic function (4,8,18,26). Bilateral excitotoxic lesions of the magnocellular basal nucleus (MBN) in rodent and nonhuman primates have been considered as a useful animal model in mimicking the cholinergic and memory deficits seen in AD (17,19).

Most lesion studies have focused on biochemical effects (5,12,31,34) or on behavioral consequences of experimental degeneration of the cortical cholinergic innervation (25,29), and only minor attention was given to the cholinergic fibers remaining intact within the denervated cortical areas. In AD, abnormal, swollen, and tortuous axonal processes containing acetylcholinesterase (AChE) (30) or choline acetyltransferase (ChAT) (2,20) have been observed, frequently associated with amyloid deposit in the senile plaque. The axonal malformations seen in AD were not seen after MBN lesions in adult rodents (30) which represents an anatomical discrepancy between the animal model and AD. Aberrant cholinergic fibers, although not associated with amyloid, do occur in nonhuman primates in normal aging (9,33) and were occasionally observed in the cortex of senile rats (3). These findings indicate that the use of aged animals in the lesion model may more

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closely approximate the fate of the cortical cholinergic fibers in AD. This suggestion is further supported by a study showing the MBN lesion-induced cholinergic depletion in rats exacerbates during the aging process after an initial recovery (16).

The aim of the present investigation is to study the anatomy of the cholinergic innervation in normal aging and approximately two years following bilateral MBN lesions. This report describes morphological changes in ChAT-immunoreactive fibers remaining in the denervated cortex as a particular effect of these lesions combined with aging. These cholinergic fiber malformations are compared with those encountered in autopsy human brain tissue from AD and age-matched control subjects.

#### METHOD

##### *Animal Experiments*

Twenty male Wistar rats were used in this study. At the age of six months, 10 animals were anesthetized with sodium pentobarbital (i.p., 30 mg/kg body wt.) and Hypnorm (Duphar, i.m., 0.4 mg/kg body wt.) and received bilateral, multiple small ibotenic acid injections along the entire length of the MBN (5 times 0.5  $\mu$ g in 1  $\mu$ l saline, pH = 7.4, or 2 times 1.0  $\mu$ g in 1  $\mu$ l; see Ref. 13 for further details). These animals survived for 25 months after the lesions were made. Another group of 10 animals reached similar senile ages (30–34 months) without being exposed to surgery. All rats were deeply anesthetized (pentobarbital, 150 mg/kg body wt.) and transcardially perfused briefly with calcium-free Tyrodes, followed by a fixative solution of 4% paraformaldehyde, 0.5% glutaraldehyde, and 15% saturated picric acid in 0.1 M phosphate buffer, pH = 7.4. The brains were removed from the skull, postfixed for 2 h in cold fixative without glutaraldehyde, and stored for 24 h in 30% buffered sucrose for cryoprotection. The brains were quickly frozen in dry ice and cut into 20  $\mu$ m transverse sections on a cryostat microtome. Free floating sections were serially collected in phosphate buffered saline (PBS, 0.01 M, pH 7.4).

One series of sections was stained for AChE according to the procedure of Hedreen et al. (15). The AChE-stained sections were used for analysis of the cholinergic fiber densities in the

TABLE I

RELATIVE DENSITIES OF AChE-POSITIVE FIBERS IN LAYER III OF THE LATERAL FRONTAL AND PARIETAL NEOCORTICES OF AGE-MATCHED CONTROL RATS AND RATS WITH NUCLEUS BASALIS LESIONS.

Group	n	Average Number of Fiber Crossings (per mm grid line)*	Extreme Values	
			Lowest	Highest
Age-matched controls	9	154.3 $\pm$ 3.5	132.9	165.0
MBN-lesioned cases	9	46.8 $\pm$ 5.6†	26.0	73.7

\*Means  $\pm$  SEM.

† $p < 0.0003$ .

cortex. The relative fiber density was assessed with an ocular line grid for counting the numbers of fiber crossings in the lateral frontal and parietal cortices (14). Two grid lines were positioned in layer III of the cortex parallel to the cortical surface. Crossing fibers were counted at six positions arranged from lateral to dorsomedial neocortex in each hemisphere at three different anterior–posterior levels (frontal, anterior, and posterior parietal cortex). Counts of each animal were averaged per 1,000  $\mu$ m grid line.

Adjacent series of sections were processed for immunocytochemical detection of ChAT by using a polyclonal antihuman placental ChAT antibody raised in goat (7). All antibodies were diluted in PBS containing 0.05% triton X-100. First the sections were preincubated in 10% normal rabbit serum (Zymed) for 1 h followed by an overnight incubation in primary goat anti-ChAT (1:1000). Thereafter the sections were exposed to rabbit anti-goat IgG (1:50, Sigma, 2 h) and goat peroxidase antiperoxidase (PAP, 1:500, Dakopatts, 3 h). The peroxidase was visualized by standard processing with 0.04% DAB and 0.015% hydrogen peroxide.

Another series of sections were transferred to the silver staining procedures and processed together with the sections from the human brains (see next). In contrast to the human material, the rat brain sections showed no plaque-like staining.

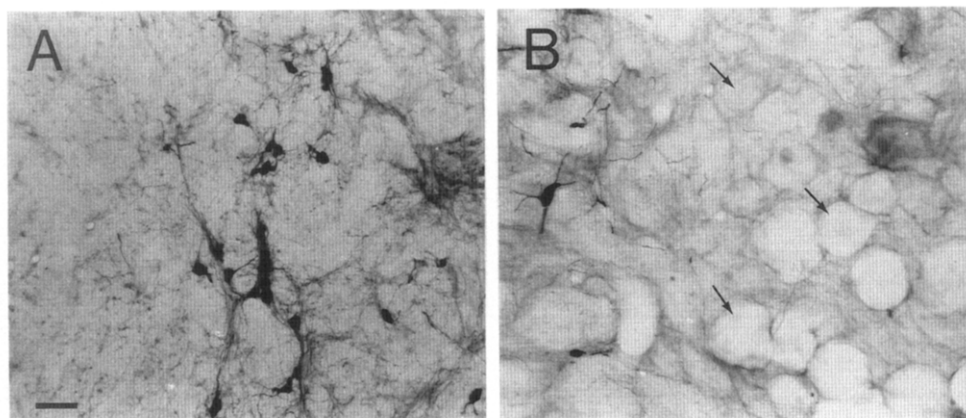


FIG. 1. Photomicrographs of the MBN/globus pallidus region of an age-matched control rat (A) and one injected 25 months earlier with ibotenic acid (B). Both sections were stained immunohistochemically for ChAT revealing the cholinergic neurons of the MBN (A). The lesioned brain has a strongly reduced number of ChAT-positive cells and contains spherical, hyaline bodies instead (B, arrows). Scale bar in A = 50  $\mu$ m; both panels have same magnification.

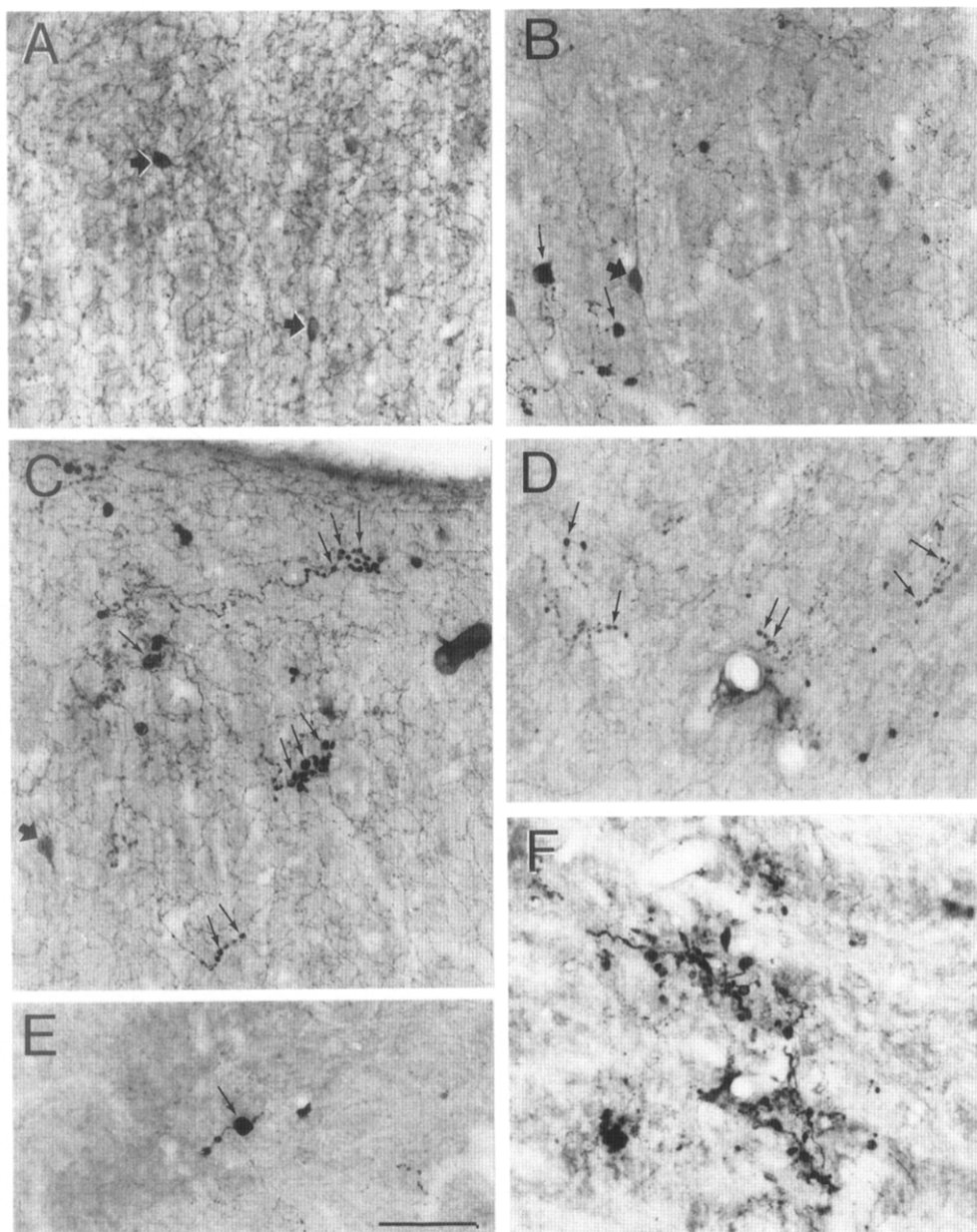


FIG. 2. Photomicrographs of choline acetyltransferase-positive fibers and bipolar interneurons (thick arrows) in the neocortex (A–C), hippocampal region (D,E), and thalamus (F) of senescent rats. (A) Normal pattern in parietal area of an age-matched control. (B) Strongly reduced number of ChAT-immunoreactive fibers in homologous area of a MBN-lesioned case. Notice intensely stained swellings (thin arrows). (C–D) Age-matched controls with abnormal cholinergic processes in the parietal cortex (C), dorsal subiculum (D), field CA2 of the hippocampus (E), and lateral geniculate region (F). Some swellings are indicated with thin arrows. Scale bar in E = 50  $\mu$ m; all panels have same magnification.

#### *Analysis of ChAT-Immunoreactive Aberrations*

Each lesioned and control animal showing clear ChAT-immunoreactivity of fibers in the cortex, hippocampus, and amygdala was admitted to the analysis of the distribution and frequency of aberrations of the cholinergic innervation. The numbers of abnormal swellings (exceeding a diameter of 3  $\mu$ m) throughout the neocortex, the olfactory, and medial mesolimbic cortices, and the hippocampal region (hippocampus, subicular, and entorhinal areas) were assessed in one series of sec-

tions per case containing every 40th section of the forebrain. Each series thus contained approximately 17 coronal sections taken regularly at intervals of 0.8 mm from the rostral frontal cortical pole to the caudal entorhinal cortex.

#### *Statistical Analysis*

For statistical analysis of AChE-stained fiber and ChAT-immunoreactive aberration densities, the nonparametric Mann-Whitney *U*-Wilcoxon rank test was used. Correlation coeffi-



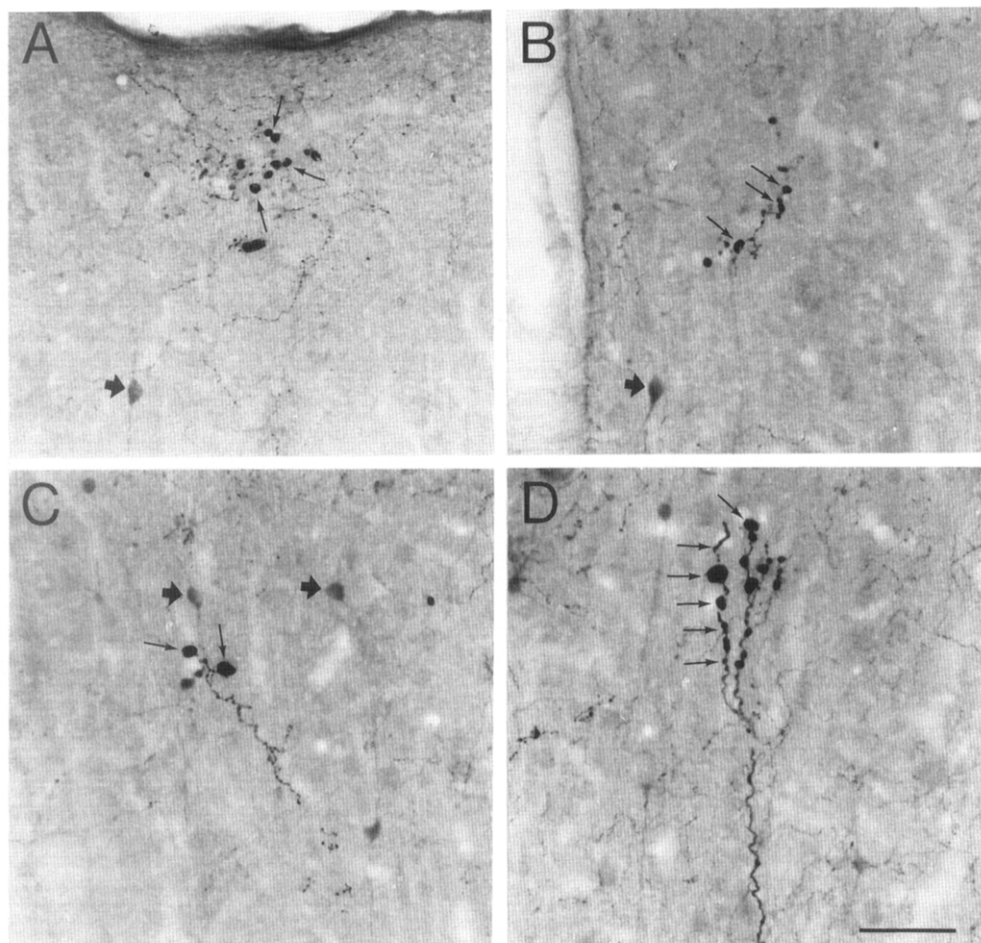


FIG. 4. Photomicrographs of ChAT-immunoreactive axonal aberrations in layers I (A), II–III (B,C) and V (D) of the frontoparietal neocortex of senescent MBN-lesioned rats. (A) Example of frequently observed grape-like clusters of globular swellings. (B–D) Multiple swellings are present along a single fiber heavily filled with immunoprecipitate. Some swellings are as large as the cell bodies of ChAT-positive interneurons (thick arrows). The density of normal-looking thin, beaded fibers is strongly reduced. Scale bar in D = 50  $\mu$ m; all panels have same magnification.

of collaterals presumably branching from a single parent axon (Fig. 2C). Clusters of distended ChAT-immunopositive processes were also present in several subcortical brain areas as the thalamic region (Fig. 2F). The frequency in which abnormalities occurred in cortex and hippocampus showed considerable variation between individual animals (Fig. 3). In case of the age-matched controls, the correlations between aberration densities in the three subdivisions of the cortical mantle reached statistical significance (Pearson's test, neocortex/piriform and mesolimbic areas:  $r = 0.85$ ,  $p < 0.002$ ; neocortex/hippocampus:  $r = 0.76$ ,  $p < 0.01$ ; piriform and mesolimbic areas/hippocampus:  $r = 0.69$ ,  $p < 0.02$ ).

**MBN-lesioned animals.** When comparing the MBN-lesioned animals with the controls, we observed similar frequency, distribution, and morphology of anomalous ChAT-immunoreactive processes in the piriform and mesolimbic cortices and the hippocampal region, areas which were not cholinergically denervated (Fig. 3). The denervated neocortex, however, showed a strongly increased number of ChAT-immunoreactive abnormalities ( $p < 0.001$ , Fig. 3) present among a strongly reduced number of normal-looking thin, beaded fi-

bers (Fig. 4A). Here, the most common type of aberration was characterized by a row of globular swellings along a thin axon, similar to those in the control rats (Fig. 4A,B). Other forms of morphological alterations included thin axons terminating in either a single swelling or in a cluster of large, irregularly shaped bulbous endings (Fig. 4C,D). Occasionally, axons were observed to be thickened along their entire length (Fig. 4D). All aberrations were heavily filled with immunoreaction product which indicates the presence of large quantities of ChAT. The abnormalities occurred in all cortical layers, although there was a slight preference for the outer layers I–III. In the MBN-lesioned cases the incidence of the abnormalities is negatively correlated with the density of AChE-positive fibers ( $p < 0.05$ ) (Fig. 5). This means that more effective lesions not only cause a greater reduction of cholinergic fibers in the neocortex but also lead to larger numbers of aberrations in the remaining fibers.

#### *AChE-Positive Fibers in the Human Cortex*

In all selected cortical areas, a reduced density of AChE-positive fibers was observed in the cortical areas of the AD

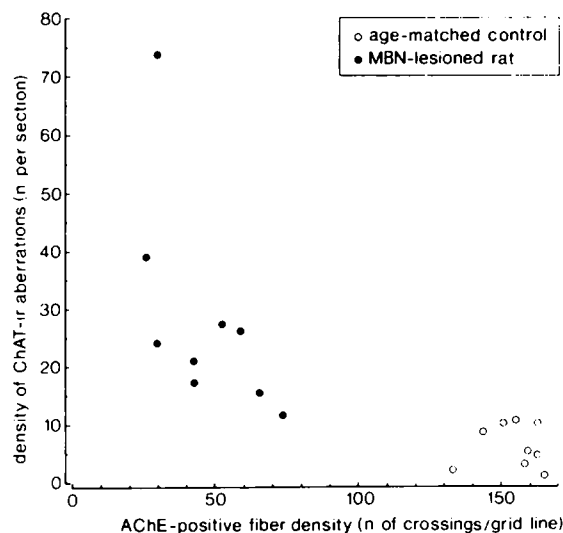


FIG. 5. Correlations between AChE+ fiber densities (number of fiber crossings per 1 mm grid line) and incidence of ChAT-immunoreactive aberrations in the neocortex. Statistical evaluation using Pearson's test:  $R = -0.013$ , ns (age-matched controls),  $R = -0.60$ ,  $p < 0.05$  (MBN-lesioned cases).

brains as compared to the control cases. Besides normal-looking fibers, several types of abnormalities could be detected in both AD and aged control brains. First, fibers with large globular swellings were present, usually in areas with lower fiber densities (Fig. 6A,B). This type of aberration was common in the AD brains but was less frequent in the normal subjects. Second, irregularly shaped swollen axonal segments or bulbous varicosities were encountered, often arranged in grape-like clusters (Fig. 6C–F). Neither in the normal nor in the AD subjects these anomalies were associated with senile plaques (compare Figs. 6C–F and Fig. 6G). Finally, we observed AChE-reactive tortuous processes present in the corona of senile plaques (Fig. 6G). The latter type of aberration, however, was only observed in the AD brains.

#### DISCUSSION

The present study shows that MBN lesions in rats in adulthood combined with a long-term survival into senescence induce a large number of malformations in the remaining cholinergic cortical fibers. These cholinergic aberrations are not exclusively associated with lesions of the MBN, but also occur, although far less frequently and less bulky, in the neocortex of the age-matched control animals. Moreover, morphologically altered cholinergic fibers in control and lesioned cases were not confined to denervated neocortical regions but appeared in medial mesolimbic and olfactory cortices, hippocampal region, and thalamus as well. The presence of swellings along ChAT-positive fibers in these areas which were not denervated and in the brains of age-matched controls is in accordance with previously reported aging-related cholinergic aberrations in the rat cortex (3). This type of aging-associated alteration is most likely not species dependent, because similar aberrations have been reported in the aged nonhuman primate brain (9,33).

The present qualitative examination of the cholinergic fiber systems in the human cerebral cortex revealed the presence of axonal malformations in the aged human brain as well. Pre-

vious reports have demonstrated distended profiles in the cortex and the hippocampus of AD patients (2,27). Our observations on the aged rat and human brains further support that these alterations are not specific features of AD, but may express a general aging-related pathology of the forebrain cholinergic system. Such distended structures likely represent a preliminary state of degeneration, although an altered attempt of axonal sprouting cannot be ruled out.

The MBN-lesioned aged rats and AD subjects bear in common not only a reduced density of cholinergic fibers in the cortex, but also increased quantities of fiber malformations (see also ref. 27). However, an important difference between the experimental rat brain and the AD brain is the association of a part of the altered cholinergic axonal processes in AD brains with amyloid deposits in senile plaques, which is not the case in the rat brain. In concert with a recent report (32), we did not succeed in revealing plaque-like or tangle-like structures in the same rat brain tissue using silver degeneration techniques (unpublished observations). Aberrant fibers in the rodent brain thus appear to be phenomena evidently independent from amyloid deposition or development of the dystrophic neurites usually shown with Gallyas silver staining (13) or Alz-50 immunolabeling (36). One complication in this respect, however, is the probability that dystrophic neurites shown by Alz-50 immunoreactivity or Gallyas staining are of dendritic origin (21). These techniques may thus not visualize distended axonal processes in rat or human brain.

Alternatively, the present results indicate that the pathogenesis of the abnormal cholinergic fibers in the rat neocortex depends both on aging and cholinergic denervation, for two reasons. First, MBN lesions in adult rats did not result in anomalies of the remaining cortical cholinergic innervation up to three months after similar MBN lesions (unpublished observations). Second, the cholinergic aberrations in the denervated neocortex clearly outnumbered those present in homologous areas of the aged control rats. Because both factors, cholinergic denervation and aging, also occur in AD, one may speculate that the development of abnormal distended cholinergic fibers in the human cortex in the advanced stage of the disease is not solely due to senile plaque formation, but may partly be dependent on the cholinergic denervation.

An explanation for the strong increase of fiber anomalies in the experimentally denervated rat cortex may be found in the delayed onset of other deleterious changes of the surviving MBN neurons in the vicinity of the lesion area. Regarding the correlation between lesion efficacy (reflected in the reduction of fiber density) and incidence of aberrations, these delayed changes are somehow related to the lesion and may underlie increased susceptibility of the surviving cholinergic cells in the vicinity of the lesion area for degeneration in senescence. In line with this suggestion, Höhmann et al. (16) observed a reoccurring decline in neocortical ChAT activity as a long-term effect of MBN-lesions (12 vs. 3 month post-lesion). Interestingly, these authors described the presence of spherical, hyaline accretions in the brains of 12-months post-lesion animals but not in animals 3- and 9-months post-lesion. Similar hyaline bodies, presumably representing end-stage of toxin-induced degeneration, were found in the MBN region in all aged, lesioned animals used in the present study. The delayed pathogenic mechanisms in the lesion area may coincide with cell degeneration that results from the aging process, so adding to the typical lesion- and aging-related axonal aberrations in the cortical projection area. In this respect, the acute lesions as performed in the rat model differ most likely from neuronal degeneration in the nucleus basalis of Meynert in AD. Breakdown of this nu-



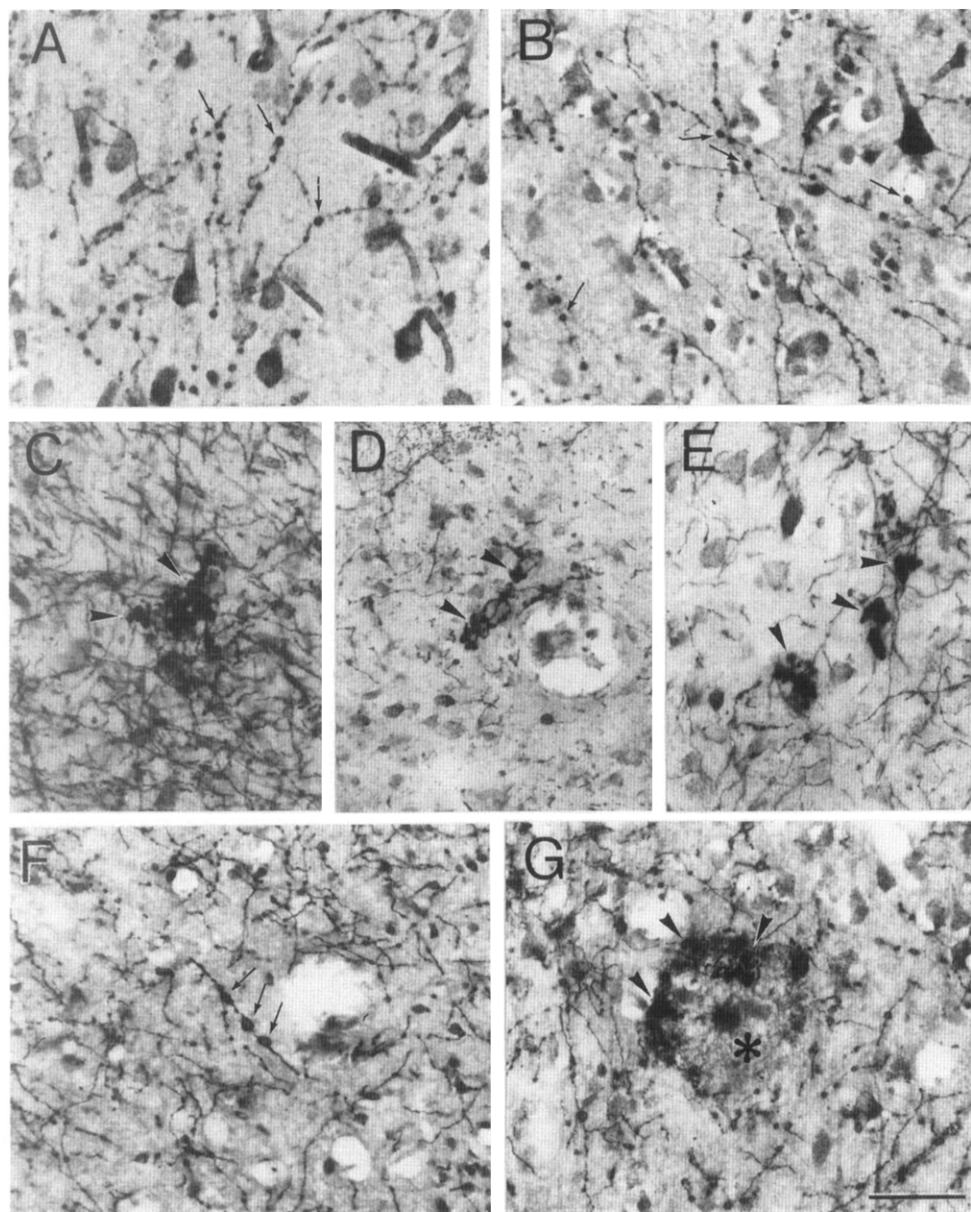


FIG. 6. Photomicrographs of AChE-positive structures in human cortical tissue. (A,B) AChE-positive fibers exhibiting enlarged globular swellings in the postcentral gyrus of an aged control subject (A) and an AD patient (B). (C-E) Grape-like clusters of swellings in the entorhinal (C,E) and frontal cortex (D) of an aged control (C) and an AD subject (D,E). There is no sign of the presence of a senile plaque near these swellings (compare with G). (F) Multiple swellings (thin arrows) along a fiber in the temporal lobe of an AD subject. (G) Swollen AChE-positive axonal processes (thin arrows) in the corona of a senile plaque (asterisk) in the frontal cortex of an AD subject. The senile plaques can easily be distinguished in the AChE-stained sections. Scale bar in G = 50  $\mu$ m; all panels have same magnification.

cleus in AD likely develops gradually and coincides with neurofibrillary changes (28) and extracellular  $\beta$ -amyloid deposition in plaques (1). The at present unknown mechanisms leading to fiber malformations in the aged, MBN-lesioned rats may therefore substantially differ from those causing fiber pathology in AD, especially in the case of interference with senile plaque formation. In aged nonhuman primates, however, it was shown that aberrant fibers and  $\beta$ -amyloid deposition do not match (9). Such a possible dissociation in humans with AD

has received little attention but may be important in the understanding of the mechanisms of disintegration of the fore-brain cholinergic system in AD.

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